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**RESEARCH ARTICLE**

# Atoh8: a transcriptional regulator of *HAMP* is down-regulated in HuH7 cells cultured in the β-thalassemia patient sera and β-thalassemic mice

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## ABSTRACT

**Objective:** This study aimed to demonstrate the down-regulation of Atoh8 relating to hepcidin suppression in β-thalassemia. **Methods:** HuH7 cells were cultured in DMEM medium supplemented with 20% β-thalassemia patient sera (HbE/β-thalassemia and β-thalassemia major) or normal serum as a control. To investigate the effect of Atoh8 on *HAMP* transcription in β-thalassemia, HEK293 cells were transfected with ATOH8-FLAG expression plasmid and cultured in DMEM medium supplemented with 20% β-thalassemia patient sera. *Atoh8* and *HAMP* mRNA expression were analyzed by qPCR, and *HAMP* promoter activity was detected by the luciferase reporter assay. In addition, hepatic *Atoh8* and *Hamp1* mRNA expression in β-thalassemic mice were also determined. **Results:** In the cultured HuH7 cells, *HAMP* mRNA levels and *HAMP* promoter activity in all β-thalassemia serum groups were significantly lower than the normal serum group. Interestingly, our study found that Atoh8 expression was down-regulated in the same direction as *HAMP* expression being suppressed despite iron overload. The Atoh8 transfection could enhance *HAMP* expression in endogenous *HAMP* mRNA levels as well as *HAMP* promoter activity in HEK293 cells cultured in the β-thalassemia patient sera. In accordance with this, hepatic *Atoh8* and *Hamp1* mRNA expression in β-thalassemic mice were also down-regulated. **Conclusion:** It's likely that the increased erythropoietic activity in β-thalassemia would suppress Atoh8 expression, subsequently down-regulating hepcidin expression. Therefore, Atoh8 would be a novel regulator of *HAMP* transcription in the β-thalassemia.



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## 1. Introduction

Hepcidin, an iron regulatory hormone, is a 25-amino acid peptide produced by hepatocytes in the liver (1-6). It plays a critical role in the balance of systemic iron homeostasis by inducing the internalization and degradation of the iron- exporter ferroportin on enterocytes, hepatocytes and macrophages (7-11). Thus the increase of hepcidin inhibits duodenal iron absorption and iron release form hepatocytes and macrophages. This can limit iron overload; however, urinary and serum hepcidin levels in β-thalassemia are reduced (12-15) leading to increased iron absorption which exacerbates iron overload condition.

Hepatic hepcidin (*HAMP*) mRNA expression is regulated in by three major stimuli including iron levels, inflammation and erythropoietic activity via different signal transduction pathways. The regulation of *HAMP* transcription responds to changes of tissue iron though the bone morphogenic protein (BMP) signaling pathway and a change of hepatic BMP6 levels (16, 17). The *HAMP* transcription is activated by the inflammation via STAT-3 signaling pathway (18). The signal transduction pathway to regulate *HAMP* transcription under the erythropoietic activity remains unclear. Two molecules produced by erythroid precursors, the growth differentiation factor 15 (GDF15) and the twisted gastrulation factor 1 (TWSG1), and the bone morphogenetic protein binding endothelial cell precursor-derived regulator (BMPER) produced by endothelial cells have been reported as the negative regulator of *HAMP* transcription by BMP signaling pathway (19-22). In addition, a recent study suggested that erythroferrone (ERFE), produced by erythroid precursors, acts as a new erythroid-derived hormone and inhibits hepcidin expression during stress erythropoiesis (23). In β-thalassemia studies, Tanno et al demonstrated that GDF15 levels were elevated in β-thalassemia patient serum with low hepcidin and that GDF15 suppressed hepcidin mRNA expression in human hepatocyte cultures (19). In contrast, Casanovas et al

reported that the hepcidin expression was suppressed in Gdf-15$^{-/-}$ mice subjected to phlebotomy and that bone marrow GDF15 were not increased in the β-thalassemic mice (24). Therefore, the relationship between GDF15 and hepcidin regulation remains controversial. TWSG1 interfered with BMP-mediated hepcidin expression in human hepatocyte cultures and was up-regulated in the β-thalassemic mice (20). ERFE mRNA expression was increased in the β-thalassemic mice, and hepcidin suppression in ERFE-deficient mice was failed after hemorrhage (23). However, human correlative studies of TWSG1 and ERFE have not been investigated yet.

Recently, Patel et al (25) has identified Atoh8, atonal homolog 8 (Drosophila), as a novel positive regulator of *HAMP* transcription via two independent mechanisms: E-box dependent transcriptional activation; and the increase of cellular pSMAD1,5,8 levels in BMP signaling pathway. In addition, the down-regulation of Atoh8 was observed in mice with increased erythropoietic activity, which appears to override the activation of Atoh8 by the increased iron levels. All of these explain how hepcidin suppression was carried on by increased erythropoietic activity in the conditions of liver iron loading and increased BMP6 levels without any change in pSMAD1,5,8 levels (26). Given that the increased erythropoiesis activity suppressed Atoh8 expression similar to hepcidin expression we hypothesized that Atoh8 may be a novel candidate transcriptional regulator of *HAMP* in the β-thalassemia.

## 2. Materials and Methods

### *2.1. β-Thalassemia serum samples*

10-18 years old male (N=11) and female (N=12) HbE/β-thalassemia patients (HbE/T-Male and HbE/T-Female) and male (N=9) and female (N=6) β-thalassemia major patients (TM-Male and TM-Female) from Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand were the

volunteers for this research. The β-thalassemia patients are usually treated with iron chelator(s) (DFP, DFO or DFX) and regularly receive blood transfusion. The experimental protocol was conducted with the approval of the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (Research ID: 2151/ Study Code: BIO-2556-02151). Their bloods were collected monthly for 3 months before they receive the blood transfusion. The bloods were collected into 5-ml vacuum tubes, left to clot at room temperature for 30 minutes and centrifuged at 3,000g for 10 minutes. The sera were collected at stored at -20ºC until use. All sera in the same group were pooled together and blood iron parameters were determined. Serum Iron (SI) and Total Iron Binding Capacity (TIBC) were analyzed by Clinical Chemistry Laboratory, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. Non-transferrin bound iron (NTBI) concentrations were measured by using NTA chelation/HPLC method (27). Serum ferritin levels were analyzed by Tumor Maker Laboratory, Faculty of Medicine, Chiang Mai University, Thailand. Serum hepcidin levels were estimated by using Hepcidin (Hepc) ELISA Kit (CEB979Hu, Uscn Life Science Inc., Hubei, China).

### *2.2. Cell culture and cell transfection*

#### *2.2.1. Cell culture*

Human hepatoma cell lines (HuH7) or human embryonic kidney 293 cell lines (HEK293) were cultured in DMEM medium supplemented with 10% (*v/v*) fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin-streptomycin and incubated at 37°C under a humidified 5% $CO_2$ atmospheric condition.

The conditions for culturing cells in human sera were determined to imitate β-thalassemia patients as previously described (28). 24-hour (60% confluent) grown cells were starved in serum-free DMEM medium supplemented with 1% penicillin-streptomycin for 24 hours as a starvation

step. Subsequently, the medium was removed and the cells were grown in DMEM medium supplemented with 20% human sera [Normal (Invitrogen, UK), male HbE/ β-thalassemia, female HbE/ β-thalassemia, male β-thalassemia major and female β-thalassemia major], 2 mM L-glutamine and 1% penicillin-streptomycin for 24 hours. *HAMP* and *Atoh8* mRNA expression were analyzed by qPCR.

### *2.2.2. Cell transfection*

#### *2.2.2.1.* **HAMP** *promoter transfection in HuH7 cells*

To confirm endogenous *HAMP* mRNA levels from qPCR result, the *HAMP* promoter assay was performed. Before the cells were grown in DMEM medium supplemented with human sera, *HAMP* reporter plasmid was co-transfected with a TK-renilla control reporter plasmid at a 1:4 ratio in serum-free DMEM medium supplemented with 1% penicillin-streptomycin into the HuH7 cells for 24 hours in the starvation step as mentioned above by using Fugene 6 (Promaga, UK) according to the manufacturer's instructions (29). After the transfected cells were grown in DMEM medium supplemented with human sera, *HAMP* promoter activity was detected by luciferase reporter assay.

#### *2.2.2.2. Atoh8 and HAMP promoter transfection in HEK293 cells*

To study the effect of Atoh8 on *HAMP* transcription, HEK293 cells, known as the model for Atoh8 transfection as the previous study (25), were transfected with ATOH8-FLAG expression plasmid or empty plasmid (Origen vector) in serum-free DMEM medium supplemented with 1% penicillin-streptomycin for 6 hours in the starvation step as mentioned above by using Fugene 6. After the transfected cells were grown in DMEM medium supplemented with human sera, *Atoh8* mRNA and *HAMP* mRNA expression were determined by qPCR.

For *HAMP* promoter assay, *HAMP* reporter plasmid was co-transfected with a TK-renilla control reporter plasmid (1:4 ratio) and ATOH8-FLAG expression plasmid in serum-free DMEM

medium supplemented with 1% penicillin-streptomycin into the HEK293 cells for 6 hours in the starvation step by using Fugene 6 (25). After the transfected cells were grown in DMEM medium supplemented with human sera, *HAMP* promoter activity was detected by the luciferase reporter assay.

### *2.3. Animals*

7-8 months old male (N=5) and female (N=5) C57/BL6, wild type (WT, $^{mu}\beta^{+/+}$) and heterozygous β-globin knockout (BKO, $^{mu}\beta^{th\text{-}3/+}$), were kindly supplied by Thalassemia Research Center, Institute of Molecular Bioscience, Mahidol University, Salaya Campus, Thailand (30, 31) and conducted under the Animal Ethical Committee of Medical Faculty, Chiang Mai University, Thailand (Reference Number 42/2556). The mice were sacrificed and their livers were collected to determine hepcidin1 (*Hamp1*) and *Atoh8* mRNA expression by qPCR.

### *2.4. RNA extraction and cDNA synthesis*

RNA was extracted from HuH7 cells or HEK293 cells by using TRIzol reagent (Invitrogen, UK). In mice, RNA was extracted from 100 mg of liver by using TRIzol and the genomic DNA was removed by using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. 1 μg of RNA was reverse transcribed into complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA).

### *2.5. Quantitative real-time PCR (qPCR)*

#### *2.5.1. In cell culture*

The qPCR reactions of cDNA were performed using TaqMan Gene Expression Assay (Applied Biosystems) on the ABI PRISM 7900 HT PCR machine (Applied Biosystems, Paisley, UK) or ABI 7500 fast real-time PCR machine (Applied Biosystems, Paisley, UK) according to the

manufacturer's protocol. mRNA expression of *HAMP* (Hs00221783_m1) or *Atoh8* (Hs01031629_m1) was normalized to housekeeping gene *RLP19* (Hs01577060_gH) and expressed as a fold change by using ΔΔCT method (32).

#### *2.5.2. In mice*

The qPCR reactions of cDNA were performed using EXPRESS SYBR® GreenER™ qPCR Supermix Universal Kits (Invitrogen, Carlsbad, CA, USA) on the ABI 7500 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The primer sequences used in qPCR are presented as follows; *mHamp1* forward: CCTGAGCAGCACCACCTATC, *mHamp1* reverse: TGCAACAGATACCACACTGGG, *mAtoh8* forward: TCAGCTTCTCCGAGTGTGTG, *mAtoh8* reverse: TAGCCTGTGGCAGGTCACT (25), *mActb* forward: GGTCCACACCCGCCAC, *mActb* reverse: GTCCTTCTGACCCATTCCCA. mRNA expression of *Hamp1* or *Atoh8* was normalized to housekeeping gene β-actin (*Actb*) and expressed as a fold change by using ΔΔCT method (32).

### *2.6. Luciferase reporter assays*

The luminescence from HuH7 cells or HEK293 cells was detected by using Dual-Luciferase Reporter Assay according to the manufacturer's instructions (Promaga, UK) and measured by a luminometer (Promaga, UK). Relative luciferase activity was calculated from the ratio of the signal of firefly luciferase to TK-renilla luciferase and expressed as the fold change to the control group.

### *2.7. Statistical Analysis*

The data are presented as means±SD. Data analysis was undertaken by using SPSS program, and statistical significance was determined by using one-way analysis of variance (ANOVA). The p value $<0.05$ was considered significant.

## 3. Results

### *3.1. Blood iron parameters*

The blood iron parameters of the pooled human sera: normal, male HbE/ β-thalassemia, female HbE/ β-thalassemia, male β-thalassemia major and female β-thalassemia major were analyzed as shown in the **Table 1**. Serum iron, transferrin saturation, plasma NTBI and plasma ferritin levels in all β-thalassemia patient sera were higher than those in normal serum although plasma hepcidin levels were not different, suggesting that the thalassemia patient sera have iron overload condition.

### *3.2. Effect of β-thalassemia patient sera on HAMP and Atoh8 expression in HuH7 cells*

After 24-hour (60% confluent) grown HuH7 cells were starved in serum-free DMEM medium for 24 hours, HuH7 cells were cultured in DMEM medium supplemented with 20% human sera: normal, male HbE/ β-thalassemia, female HbE/ β-thalassemia, male β-thalassemia major and female β-thalassemia major for 24 hours. Subsequently, *HAMP* and *Atoh8* mRNA expression were detected by qPCR. *HAMP* **(Figure 1A)** and *Atoh8* **(Figure 1B)** mRNA levels and *HAMP* promoter activity **(Figure 2)** in all β-thalassemia serum groups were significantly lower than those in the normal serum group, and there was no difference between genders. Interestingly, our study demonstrated that β-thalassemia patients have not only the decreased *HAMP* expression but also the reduced Atoh8 expression.

### *3.3. Hepatic Hamp1 and Atoh8 mRNA expression in β-thalassemic mice*

Hepatic *Hamp1* **(Figure 3A)** and *Atoh8* **(Figure 3B)** mRNA levels in BKO mice were lower than those in WT mice but significantly in female. However, the *Hamp1* and *Atoh8* mRNA expression were not different between genders.

### *3.4. Effect of Atoh8 transfection on HAMP expression in HEK293 cells grown in β-thalassemia patient sera*

To determine whether Atoh8 regulates *HAMP* transcription in β-thalassemia, HEK293 cells were transfected with increasing amounts of the ATOH8-FLAG expression plasmid and grown in the β-thalassemia patient sera. The HEK293 cells were transfected with 0.5, 1, 2 and 4 ug ATOH8-FLAG expression plasmid and grown in DMEM medium containing 20% female HbE/ β-thalassemia serum for 24 hours. The Atoh8 transfection could significantly induce *Atoh8* mRNA expression **(Figure 4A)** and up-regulate *HAMP* mRNA expression **(Figure 4B)** in the dose-dependent manner at 2 and 4 µg ATOH8-FLAG expression plasmid. In accordance with endogenous *HAMP* mRNA levels, *HAMP* promoter activity was more sensitive and significantly increased in the dose-dependent manner with 0.5, 1 and 2 µg ATOH8-FLAG expression plasmid **(Figure 5)**. Furthermore, the Atoh8 transfection could significantly and markedly enhance *HAMP* expression in both endogenous *HAMP* mRNA and *HAMP* promoter activity not only in the female HbE/ β-thalassemia serum group but also in normal, male HbE/ β-thalassemia, male β-thalassemia major and female β-thalassemia major serum group **(Figure 6 and 7)**. The empty plasmid (Origen vector) did not effect on *Atoh8* **(Figure 6A)** and *HAMP* **(Figure 6B)** mRNA levels as well as *HAMP* promoter activity **(Figure 7A)** when compared to the untransfected ATOH8-FLAG group. Thus the Atoh8 transfection could up-regulate the expression of Atoh8 probably subsequently activating *HAMP* expression.

## 4. Discussion

The down-regulation of hepcidin expression in β-thalassemic mice (33-38) and the low hepcidin levels in β-thalassemia patients (12-15, 39) have been previously reported. Although our

study showed the indifference of hepcidin levels between β-thalassemia patient serum and normal serum as the previous studies (15, 40, 41), HepG2 grown in the medium containg the thalassemia sera revealed the down-regulation of hepcidin expression (28, 40). Interestingly, this study demonstrated that not only hepcidin but also Atoh8 was down-regulated in β-thalassemia. The decrease of *Atoh8* mRNA levels also related to the reduction of *HAMP* expression, endogenous *HAMP* mRNA and *HAMP* promoter activity, in the HuH7 cells cultured in DMEM medium supplemented with 20% β-thalassemia patient sera for 24 hours. In this condition, erythropoietic regulatory proteins still effect on *HAMP* expression as same as in the patients. According to the suppression of hepatic Atoh8 and hepcidin expression in the mice under the conditions with an increased erythropoiesis activity (e.g. hemolytic anemia, hypoxia, erythropoietin treatment, and hypotransferrinaemia) (21, 25, 42-46), this study also showed the lower hepatic *Atho8* mRNA levels related to the decreased hepatic *Hamp1* mRNA levels in β-thalassemic mice. Courselaud and colleagues demonstrated that hepcidin expression varied between genders of mice (47), but not in some study (41) as well as our data Atho8 and hepcidin expression in the β-thalassemia patient serum groups and in the β-thalassemic mice were not different between genders. In addition to erythropoietic activity, Atoh8 expression responded to a change of iron levels. Hepatic *Atoh8* and *Hamp1* mRNA levels were increased in mice fed with an iron supplemented-diet and in mice treated with holo-transferrin, whereas those were decreased in mice fed with an iron deficient-diet (25, 48). Conversely, *Atoh8* and *HAMP* mRNA expression were suppressed in HuH7 cells cultured in the medium containing the β-thalassemia patient sera even though their serum iron and transferrin saturation levels are very high. Similarly, in hypotransferrinaemic (HPX) mice with high degree of chronic anemia and liver iron overload, hepatic Atoh8 and *Hamp1* expression were also down-regulated (21, 25). *Atoh8* mRNA expression was also inhibited in *Hamp1*$^{-/-}$ mice

after PHZ treatment although their serum iron was still high (25, 44). As previously shown, it is likely that the up-regulation of Atoh8 by iron was overridden by the increased erythropoiesis activity similar to the regulation of hepcidin. Therefore, in β-thalassemia Atoh8 expression was suppressed by the increased erythropoiesis activity in a similar manner and direction to hepcidin expression.

Furthermore, Atoh8 expression was also suppressed in *Hamp1*$^{-/-}$ mice after PHZ treatment increasing erythropoiesis activity, suggesting that Atoh8 is an upstream regulator of *Hamp1* (25). In HEK293 cells, Atho8 could stimulate *HAMP* transcription in two ways, directly via E-boxes and indirectly through increased pSMAD1,5,8 levels (25). In this study, Atoh8 transfection in HEK293 cultured in the medium containing the β-thalassemia patient sera could induce the *HAMP* expression in both endogenous mRNA and promoter activity. It suggested that the down-regulation of Atoh8 expression in β-thalassemia resulted from the increased erythropoietic activity and subsequently leaded to the decrease of *HAMP* expression. Therefore, in β-thalassemia Atoh8 may be a novel transcriptional regulator of *HAMP*, which is responsive to the erythropoietic activity. This is a new research for therapies and management in β-thalassemia patients.

## Conflict of Interests

The authors attest to having no conflicting interests.

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**Table 1** Blood iron parameters of human sera

| Blood chemistry | Normal | HbE/T-Male | HbE/T-Female | TM-Male | TM-Female |
|---|---|---|---|---|---|
| SI (µg/dl) | 52 | 140 | 135 | 161 | 155 |
| TIBC (µg/dl) | 339 | 148 | 135 | 161 | 155 |
| Transferrin saturation (%) | 15.34 | 94.59 | 100 | 100 | 100 |
| NTBI (µM) | -0.39 | 7.31 | 7.77 | 5.46 | 4.07 |
| Ferritin (ng/ml) | 26.31 | 1307 | 1812 | 2000 | 1405 |
| Hepcidin (ng/ml) | 34.69 | 42.78 | 38.08 | 32.90 | 47.76 |

## Figure legends

**Figure 1 Effect of β-thalassemia patient sera on *HAMP* and *Atoh8* mRNA expression in HuH7 cells grown in DMEM medium supplemented with 20% human sera for 24 hours.** Relative *HAMP* **(A)** and *Atoh8* **(B)** mRNA expression from qPCR analysis were acquired by normalizing *HAMP* mRNA or *Atoh8* mRNA to *RPL19* mRNA. The samples were determined in quadruplicate in two independent experiments. Values are expressed as mean±SD derived from a single experiment with four biological replicates for the fold change as compared to the normal serum group. The experiment shown is representative of two similar experiments. *$p<0.05$ when compared with the normal serum group.

**Figure 2 Effect of β-thalassemia patient sera on *HAMP* promoter activity in HuH7 cells co-transfected with *HAMP* reporter plasmid and TK-renilla and grown in DMEM medium supplemented with 20% human sera for 24 hours.** Promoter activity was expressed as the relative luciferase activity to the normal serum group. The samples were determined in triplicate. Values are expressed as mean±SD for the fold change as compared to the normal serum group. *$p<0.05$ when compared with the normal serum group.

**Figure 3 Hepatic *Hamp1* and *Atoh8* mRNA expression of male (N=5) and female (N=5) mice.** Relative *Hamp1* (A) and *Atoh8* (B) mRNA expression in WT and BKO mice from qPCR analysis were acquired by normalizing *Hamp1* mRNA or *Atoh8* mRNA to *Actb* mRNA. Vales are expressed as mean±SD for the fold change as compared to the WT-male group.

**Figure 4 *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (1, 2 and 4 µg) ATOH8-FLAG expression plasmid and grown in DMEM medium supplemented with 20% female HbE/ β-thalassemia serum for 24 hours.** Relative *Atoh8* **(A)** and *HAMP* **(B)** mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP*

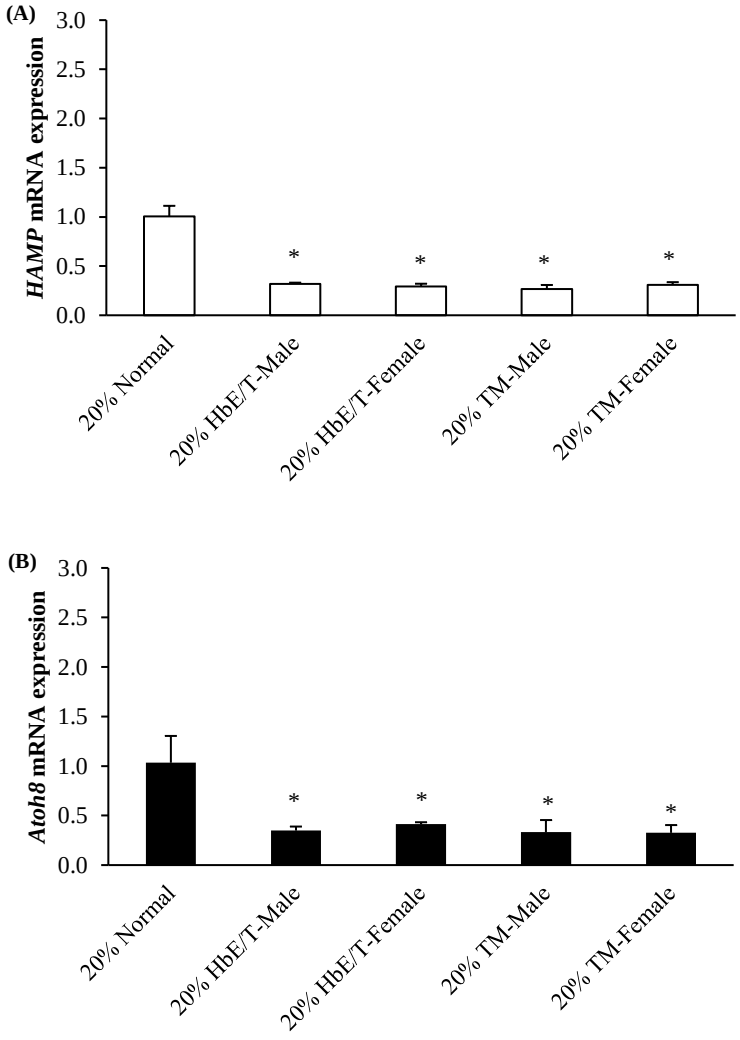
mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05 when compared with the untransfected ATOH8-FLAG group.

**Figure 5 *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter plasmid, TK-renilla and (0.5, 1 and 2 µg) ATOH8-FLAG expression plasmid and grown in DMEM medium supplemented with 20% female HbE/ β-thalassemia serum for 24 hours.** Promoter activity was expressed as the relative luciferase activity to the untransfected ATOH8-FLAG group. The samples were determined in triplicate. Values are expressed as mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group. *p<0.05 when compared with the untransfected ATOH8-FLAG group.

**Figure 6 *Atoh8* and *HAMP* mRNA expression in HEK293 cells transfected with (2 µg) ATOH8-FLAG expression plasmid or empty plasmid (Origen vector) and grown in DMEM medium supplemented with 20% human sera for 24 hours.** Relative *Atoh8* (A) and *HAMP* (B) mRNA expression from qPCR analysis were acquired by normalizing *Atoh8* mRNA or *HAMP* mRNA to *RPL19* mRNA. The samples were determined in triplicate. Values are expressed as mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group.

**Figure 7 *HAMP* promoter activity in HEK293 cells co-transfected with *HAMP* reporter plasmid, TK-renilla and (0.5 and 1 µg) ATOH8-FLAG expression plasmid (or Origen vector) and grown in DMEM medium supplemented with 20% human sera (A: Female HbE/β-thalassemia, B: Normal, C: Male HbE/β-thalassemia, D: Male β-thalassemia major, D: Female β-thalassemia major) for 24 hours.** Promoter activity was expressed as the relative luciferase activity to the untransfected ATOH8-FLAG group. The samples were determined in

triplicate. Values are expressed as mean±SD for the fold change as compared to the untransfected ATOH8-FLAG group. *$p<0.05$ when compared with the untransfected ATOH8-FLAG group.

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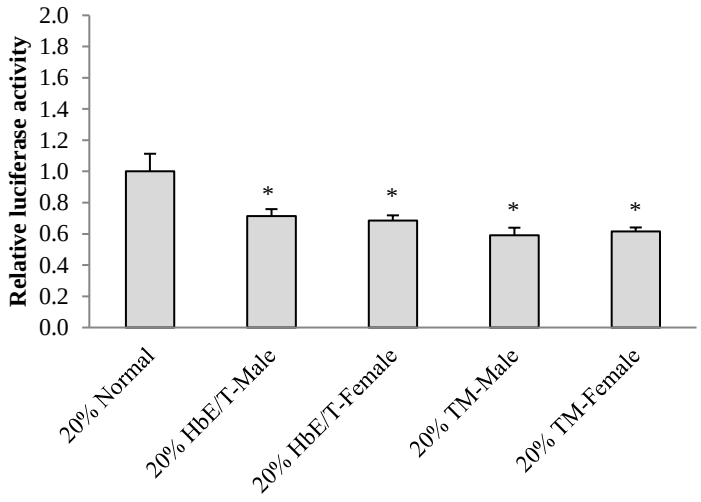


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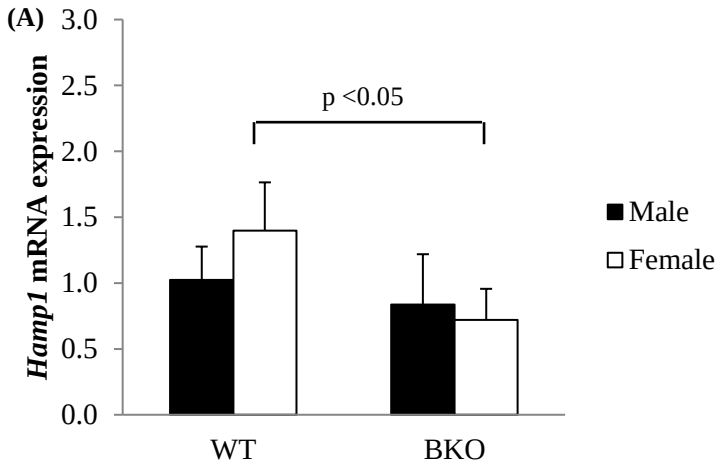


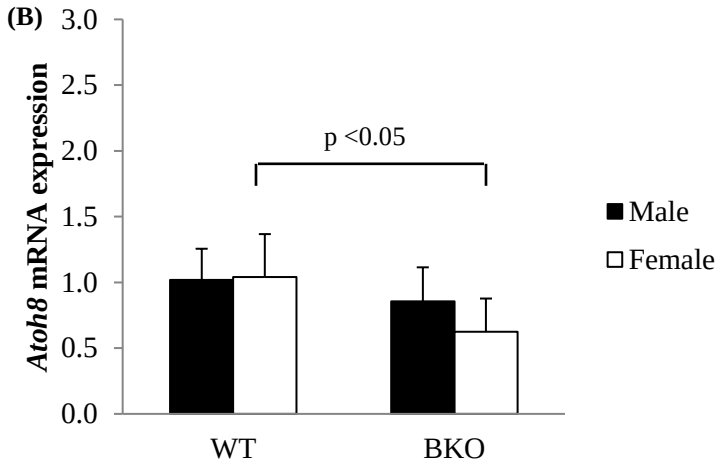


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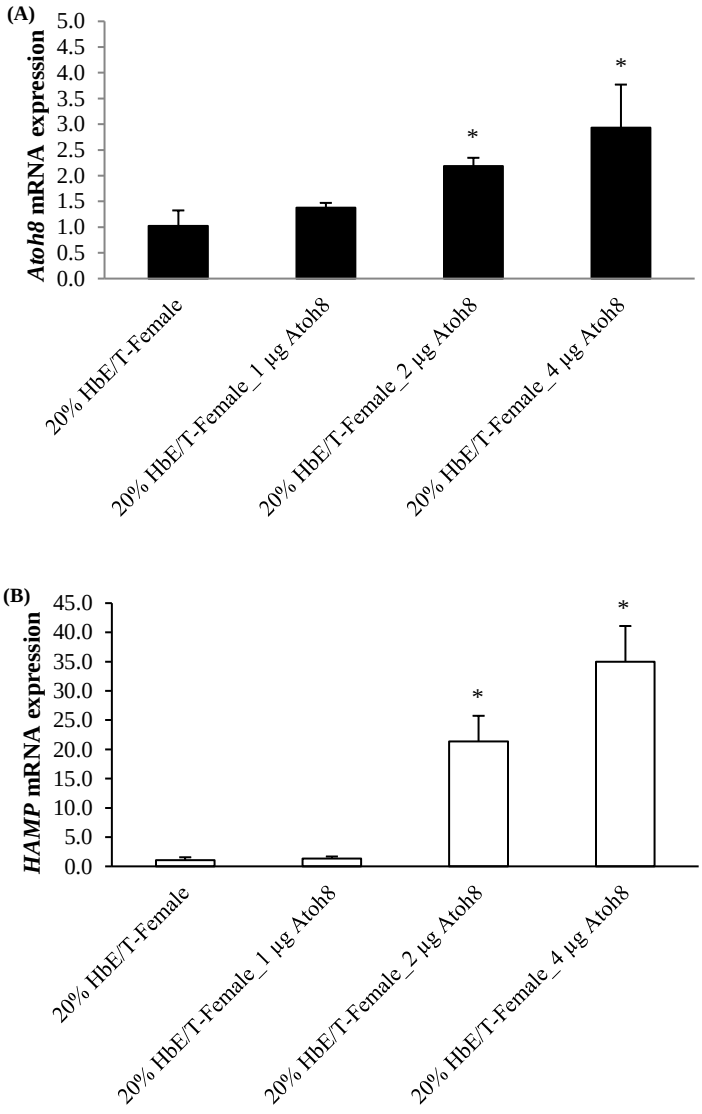


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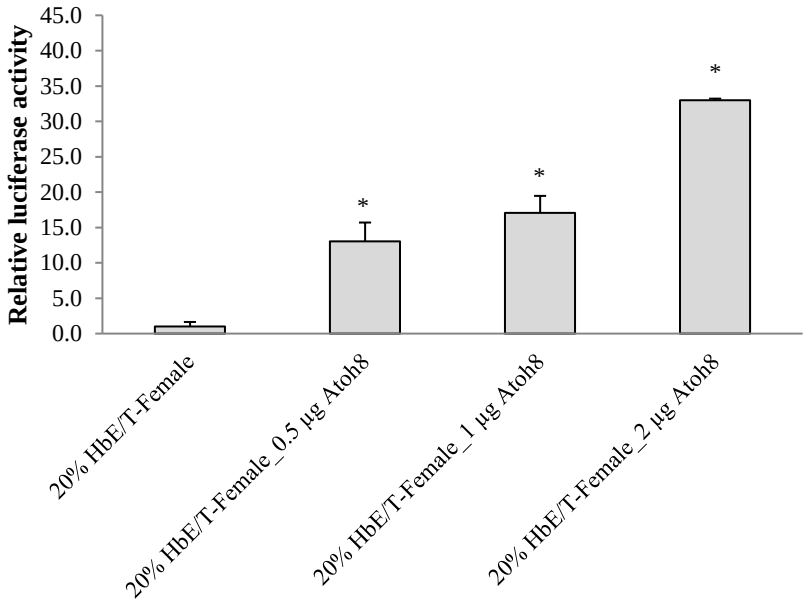


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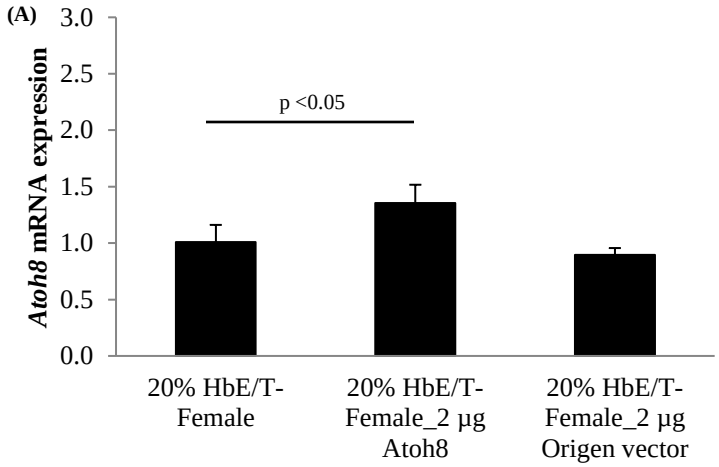


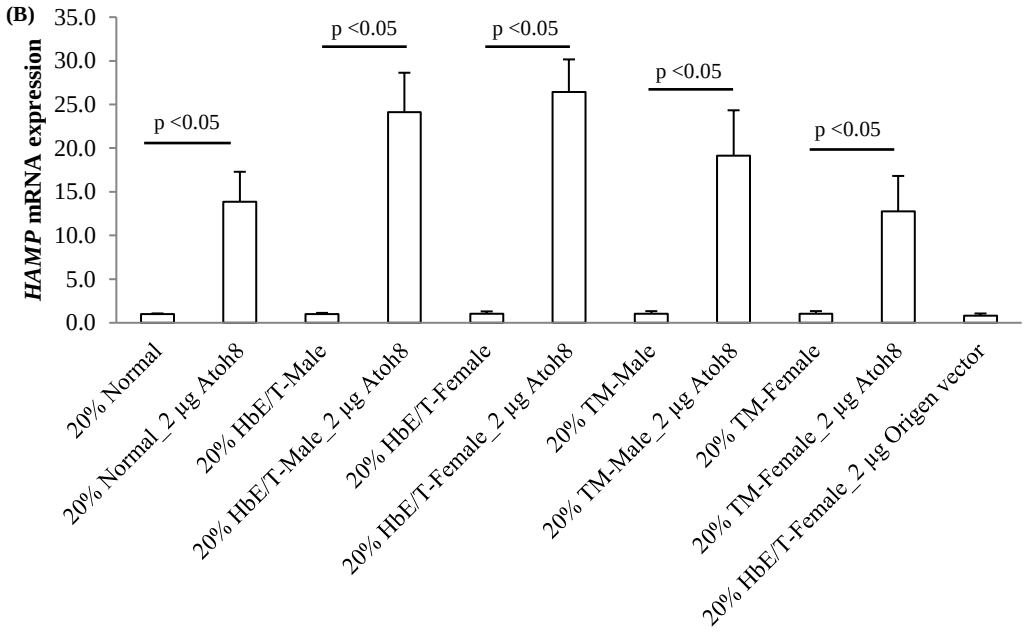


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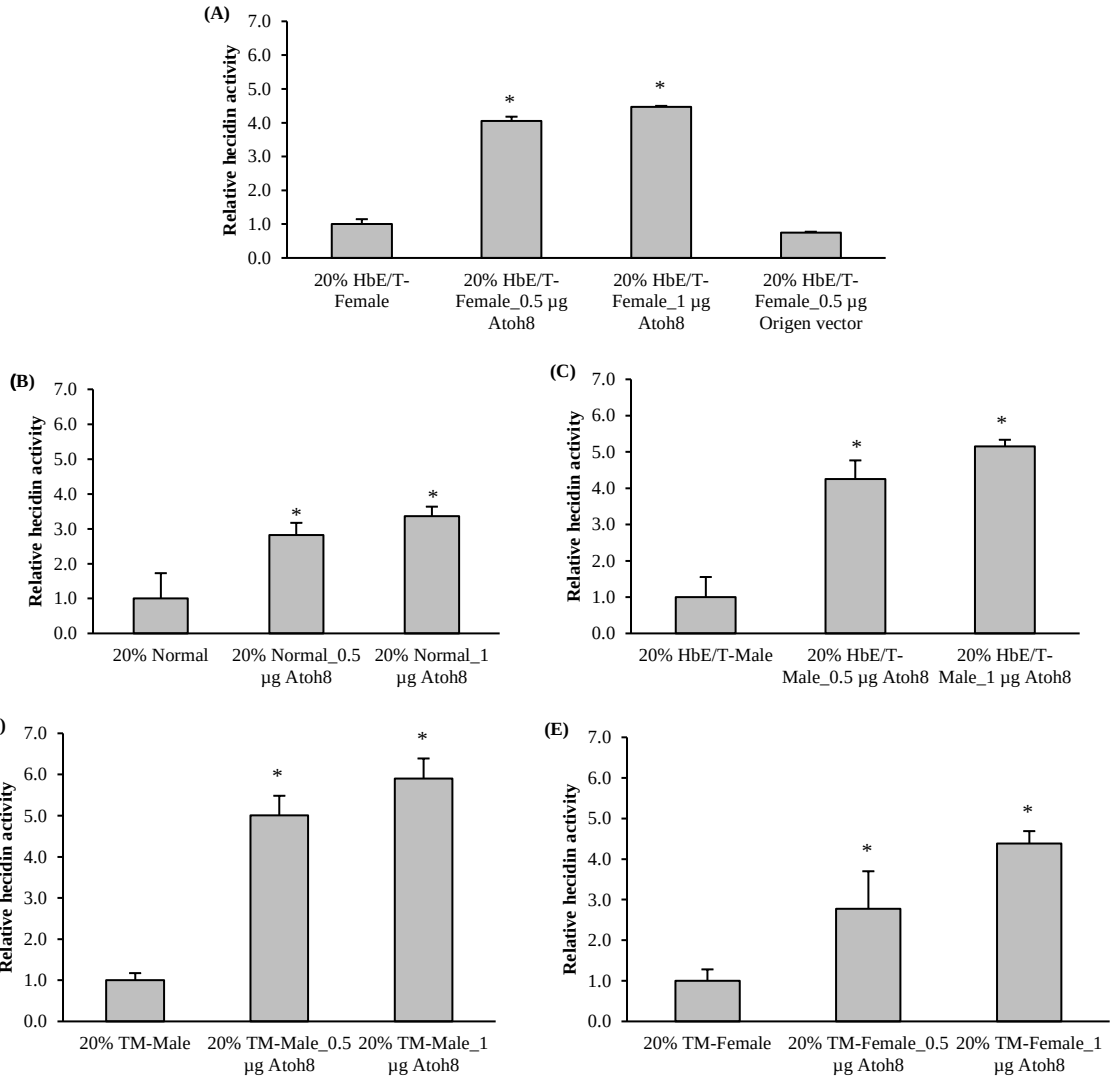


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